Center for Veterinary Biologics
and
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Testing Protocol

Supplemental Assay Method for Titration of Bovine Respiratory Syncytial Virus in Vaccines

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Table of Contents

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* assay method which employs a cell culture system utilizing cytopathic effect (CPE) to determine the bovine respiratory syncytial virus (BRSV) content of modified-live veterinary vaccines.

1.2 Keywords

Bovine respiratory syncytial virus; BRSV; potency test, CPE, titration, *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36° ± 2°C, high humidity, 5% ± 1% CO_2

2.1.2 Water bath,[2] 37° ± 1°C

2.1.3 Pipettors,[3] 25 µl and 500 µl and tips[4]

2.1.4 Vortex mixer[5]

2.1.5 Multichannel pipettor,[6] 50-300 µl x 8 or 12 channel

2.1.6 Microscope,[7] inverted light

[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750-0649 or equivalent
[2] Model MW-1120A, Blue M Electric Co., 304 Hart St., Watertown, WI 53094 or equivalent
[3] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[4] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent
[5] Vortex-3 Genie, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716 or equivalent
[6] Finnipette, Labsystems OY, Pulttitie 9, 00810 Helsinki 81, Finland or equivalent
[7] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

2.2 Reagent/supplies

2.2.1 BRSV Reference[8]

2.2.2 Embryonic bovine lung[9] (EBL) cells free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Diluent Medium

2.2.3.1 9.61 g minimum essential medium with Earle's salts without bicarbonate[10]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[11]

2.2.3.3 Dissolve with 900 ml deionized water (DW)

2.2.3.4 Add 5.0 g lactalbumin hydrolysate or edamin[12] to 10 ml of DW. Heat to 60° ± 2°C until dissolved and add to **Section 2.2.3.3** with constant mixing.

2.2.3.5 Q.S. to 1000 ml with DW, adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[13]

2.2.3.6 Sterilize through a 0.22-µm filter.[14]

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine[15]
2. 100 units/ml penicillin[16]

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[8] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[9] Available upon request from the CVB-L or equivalent
[10] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[11] Cat. No. S 5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[12] Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[13] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[14] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[15] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[16] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent.

3. 50 µg/ml gentamicin sulfate[17]
4. 100 µg/ml streptomycin[18]
5. 2.5 µg/ml amphotericin B[19]

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml MEM

2.2.4.2 Aseptically add 100 ml of gamma-irradiated fetal bovine serum (FBS).

2.2.4.3 Store at 4° ± 2°C.

2.2.5 Trypsin versene (TV) Solution

2.2.5.1 8.0 g sodium chloride (NaCl)[20]

2.2.5.2 0.40 g potassium chloride (KCL)[21]

2.2.5.3 0.58 g $NaHCO_3$

2.2.5.4 0.50 g irradiated trypsin[22]

2.2.5.5 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)[23]

2.2.5.6 1.0 g dextrose[24]

2.2.5.7 0.4 ml 0.5% phenol red[25]

2.2.5.8 QS with DW to 1000 ml.

[17] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[18] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[19] Cat. No. A-4888, Sigma Chemical Co. or equivalent
[20] Cat. No. 3624-01, J.T. Baker, Inc. or equivalent
[21] Cat. No. P217-500, Fisher Scientific Corp. or equivalent
[22] Cat. No. 0152-15-9, DIFCO Laboratories, P.O. Box 331058, Detroit, MI 48232-0758
[23] Cat. No. E 5134, Sigma Chemical Co. or equivalent
[24] Cat. No. G5146, Sigma Chemical Co. or equivalent
[25] Cat. No. P0290, Sigma Chemical Co. or equivalent

2.2.5.9 pH to 7.3 with $NaHCO_3$.

2.2.5.10 Filter through a 0.22-µm filter.

2.2.5.11 Store at -20° ± 4°C.

2.2.6 Tissue culture plates,[26] 96 well

2.2.7 Polystyrene tubes,[27] 17 x 100 mm

2.2.8 Polystyrene tubes,[28] 12 x 75 mm

2.2.9 Infectious bovine rhinotracheitis monospecific antiserum (IBR AS)[8]

2.2.10 Bovine viral diarrhea virus monospecific antiserum (BVDV AS)[8]

2.2.11 Bovine parainfluenza 3 virus monospecific antiserum (PI3V AS)[8]

2.2.12 Serological pipette,[29] 10 ml

2.2.13 Sterile graduated cylinders,[30] 25 ml, 50 ml, 100 ml, 250 ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in cell culture technique, the principles of aseptic technique, and virus titration assays.

[26] Cat. No. 3596, Corning Costar Corp. 1 Alewife Center, Cambridge, MA 02140 or equivalent
[27] Falcon® 2057, Becton Dickinson Labware 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
[28] Falcon® 2058, Becton Dickinson Labware or equivalent
[29] Falcon® 7530, Becton Dickinson Labware or equivalent
[30] Cat. No. P34546-02, P34546-03, P34546-04, P34546-05 respectively, Cole-Parmer Instrument Co., 625 Bunker Court, Vernon Hills, IL 60061-9872 or equivalent

3.2 Preparation of equipment/instrumentation

On the day of test initiation set the water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of EBL plates

3.3.1.1 Cells are prepared from healthy, confluent EBL cells, that are maintained by passing every 5 ± 2 days. On the day of test initiation, remove the cells from the growth containers by using TV Solution. Using a multichannell pipettor, add 200 µl/well of $10^{4.9}$ to $10^{5.1}$ cells/ml of EBL cells suspended in Growth Medium into all wells of a 96-well cell culture plate. Two (2) plates will test 3 Test Serials and the associated controls, These become the Test Plates. Incubate at 36° ± 2°C in a CO_2 incubator and use within 4 hr.

3.3.2 Preparation of the BRSV Reference Control

3.3.2.1 On the day of test initiation, a vial of BRSV Reference is rapidly thawed in a 36° ± 2°C water bath and tenfold dilutions made:

1. Place 4.5 ml of Dilution Medium into 5, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-5} respectively, using a 10-ml serological pipette.

2. Using a 500 µl pipettor, transfer 500 µl of BRSV Reference to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

3. Using a new pipette tip, transfer 500 µl from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing.

4. Repeat **Section 3.3.2.1.3** for each of the subsequent dilutions, transfering 500 µl from the previous dilution to the next dilution tube until the dilution series is completed (or final dilution is made).

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of test initiation, remove the seal and stopper from both the Test Serial bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer's instructions (e.g for a 50 dose container of 2 ml per dose, reconstitute with 100 ml of diluent) and aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

3.4.2 Viral neutralization. In order to determine the BRSV titer in a multifraction product, neutralize the IBR, BVDV, and PI3V fractions with monospecific AS.

3.4.2.1 IBR/BRSV Vaccine

1. 1.0 ml of reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, mix by vortexing.

2. Mix 500 µl of **Section 3.4.2.1.1** dilution of Test Serial with 500 µl IBR AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at room temperature (RT) (23° ± 2°C) for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.2 IBR/BRSV/BVDV Vaccine

1. 1.0 ml of reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, mix by vortexing.

2. 1.0 ml of **Section 3.4.2.2.1** dilution of Test Serial is mixed with 500 µl of IBR AS and 500 µl of BVDV AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.3 IBR/PI3V/BVDV/BRSV Vaccine

1. 2.0 ml of reconstituted Test Serial is diluted with 8.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, mix by vortexing.

2. 1.5 ml of **Section 3.4.2.3.1** dilution of Test Serial is mixed with 500 µl of IBR AS, 500 µl of BVDV AS and 500 µl of PI3V AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.4 BRSV Monovalent Vaccine

1. 500 µl of the reconstituted Test Serial is diluted with 4.5 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3.4.3 Sample dilutions. Four, tenfold dilutions are made from the 10^{-1} dilution of the Test Serial using Diluent Medium.

1. Place 4.5 ml of Diluent Medium into each of four 17 x 100-mm polystyrene tubes labeled 10^{-2} through 10^{-5} using a 10-ml serological pipette.

2. Pipette 500 µl of the Test Serial from the 10^{-1} tube into the 10^{-2} tube, mix by vortexing. Discard pipette tip.

3. Using a new tip each time repeat **Section 3.4.3.2** to the remaining tubes transferring 500 µl from the previous dilution tube to the next until final dilution is made (10^{-5}); mix by vortexing between each dilution.

4. Performance of the test

4.1 Inoculate 5 wells/dilution with 25 µl/well of the diluted Test Serial and the BRSV Reference Control (10^{-1} through 10^{-5}) to the Test Plate. Change tips between each unique sample (e.g., each Test Serial and the BRSV Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-1}). This becomes the BRSV Test Plate.

4.2 Maintain 5 or more wells as uninoculated cell culture controls per plate.

4.3 Incubate the BRSV Test Plate undisturbed at 36° ± 2°C in a CO_2 incubator for 126 ± 6 hr.

4.4 At the end of incubation, read the BRSV Test Plate at 100X magnification on an inverted light microscope and examine for CPE characterized by cell fusion.

4.4.1 Wells displaying 1 or more CPE foci, are considered to be positive for BRSV.

4.4.2 Results are recorded as the number of CPE positive wells versus the total number of wells examined for each dilution of the Test Serial and the BRSV Reference Control.

4.5 Calculate the BRSV endpoints of the Test Serial and the BRSV Reference Control using the method of Spearman-Kärber as commonly modified. The titers are expressed as $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) of the test wells.

Example:

10^{-2} dilution of Test Serial = 5/5 wells CPE positive
10^{-3} dilution of Test Serial = 5/5 wells CPE positive
10^{-4} dilution of Test Serial = 2/5 wells CPE positive
10^{-5} dilution of Test Serial = 0/5 wells CPE positive

Test dose titer = (X - d/2 + [d * S]) where:

X = $\log_{10}$ of lowest dilution (1)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportion of CAMs plaque positive

$$\frac{(5+3+1)}{5} = \frac{9}{5} = 1.8$$

Test dose titer = (1 - 1/2) + (1 * 1.8) = 2.3

Spearman-Kärber calculation of total CPE positive wells (12), using 5 wells = 1.9 log

Log_{10} of reciprocal dilution counted (10^{-2}) = 2.0 log

Adjust the titer to the Test Serial Dose size by adding the $\log_{10}$ of the reciprocal of the Inoculation Dose divided by the Test Serial Dose where:

Inoculation Dose = amount of diluted Test Serial inoculated onto each well

Test Serial Dose = manufacturer's recommended vaccination dose Log_{10} of reciprocal of dose factor:

$$\frac{0.025 \text{ ml inoculum}}{2 \text{ ml dose}} = \frac{0.025}{2} = 1.9 \log$$

Total = 5.8 log

Titer of the Test Serial is $10^{5.8}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 For a valid assay

5.1.1 The calculated $TCID_{50}$ titer of the BRSV Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell control wells cannot exhibit any CPE or cloudy media that would indicate contamination.

5.1.3 The lowest dilution of the BRSV Reference Control must exhibit a 100% positive CPE (5/5), and the highest (most dilute) must exhibit no positive CPE (0/5).

5.2 If the validity requirements are not met, then the assays is considered a **NO TEST** and may be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production (OP), the Test Serial is considered **SATISFACTORY**.

5.4 If the validity requirements are met but the titer of the Test Serial is less than the titer contained in the APHIS file Outline of Production the Test Serial is retested according to 9 CFR, Part 113.8.

6. Report of test results

6.1 Results are reported as $TCID_{50}$ per dose.

6.2 Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.300, U.S. Government Printing Office, Washington DC, 2000

7.2 Finney, DJ 1978. *Statistical method in biological assay*. Griffin, London. 3rd edition, pg 508.

7.3 Cottral GE, (Ed.), 1978, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg.508.

8. Sumary of revisions

This document is new and reflects current practices in the Mammalian Virology Section.